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Photoinactivation of Cultured Skin Fibroblasts by Sublethal Doses of 8-Methoxypsoralen and Long Wave Ultraviolet Light

JOACHIM POHL AND ENNO CHRISTOPHERS

Department of Dermatology, University of Kiel, Deutschland

Cultured guinea pig skin fibroblasts were treated with 8-methoxypsoralen (8-MOP) and UVA light. Determination of ^{3}H-TdR-uptake as well as counting of the number of adherent cells was carried out 24 hr later. Incubation of fibroblasts with varying concentrations of 8-MOP (10^{-4} to 10 μg/ml) or 1 to 5 J/cm^2 UVA alone showed no effect. When 8-MOP-photosensitization was followed by UVA a dose response was observed. This ranged over 3 orders of magnitude of the concentration of 8-MOP. Changes in irradiation energy produced a higher inhibition of ^{3}H-TdR incorporation as compared to changes in 8-MOP concentrations. Using the same energy of UVA changes in the length of irradiation were of no effect. Cultures in which ^{3}H-TdR uptake was inhibited by 50% showed no loss of plating activity. A fraction of these cells underwent DNA synthesis and division after reseeding. The results indicate that under the dose regimens currently used for the treatment of various skin disorders a proportion of the cells may become sublethally photoinactivated to undergo division. When reseeded these cells still are able to perform cellular functions such as spreading and attachment.

Successful photochemotherapy (PUVA) of psoriasis and other PUVA-sensitive dermatoses [1–7] is supposed to be based on interstrand cross-linking between furocoumarins and nucleic acids. This may be followed by suppression of cell division. In cultured mammalian cells treatment with 8-methoxypsoralen (8-MOP) or trimethylpsoralen and UVA produces a significant inhibition of DNA synthesis [8–11]. As under *in vivo*-conditions [12–14] this reaction appears to be dependent on the applied concentration of 8-MOP as well as the dose of light used. Information concerning the tissue level of 8-MOP after oral uptake is still lacking. Also, the minimum doses of this drug and UVA affecting DNA replication and cell growth are not precisely known. These reasons prompted us to investigate dose-responses in cultured fibroblasts.

MATERIALS AND METHODS

Preparation of Cultures

Skin fibroblasts were prepared by trypsinizing small pieces of guinea pig skin. The cells were seeded in plastic Linbro culture dishes (Linbro FB-16-24-TC; Flow Laboratories, Bonn/Germany). The growth medium was McCoy's 5A medium containing 10% calf serum, 100 IU/ml penicillin, 100 μg/ml Streptomycin (Gibco Bio-Cult, Glasgow/Scotland) and 10 μg/ml Amphotericin B (Squibb, Munich/Germany). The medium was changed every 2 days. Secondary cultures were obtained by mild trypsinization (0.25% trypsin, Gibco Bio-Cult, Glasgow/Scotland) of primary cultures. The cells were washed twice with Earle's Balanced Salt Solution (E.B.S.S., Gibco Bio-Cult, Glasgow/Scotland) and then reseeded. All cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2. They were checked for the presence of mycoplasms at regular intervals.

PUVA-Treatment

Equal volumes of 8-MOP in alcoholic solution (Basotherm, Biberach/Germany) and dimethylsulfoxide (DMSO) were mixed in order to increase the solubility of 8-MOP. This solution was diluted by adding growth medium until the final concentrations of 8-MOP (0.0001—10 μg/ml) were obtained. The concentrations of DMSO and ethanol in the growth medium did not exceed 0.37% of both in any experiment including the controls. The cultures were incubated for 1 hr at 37°C with different 8-MOP concentrations. This was immediately followed by irradiation with various doses of UVA light (0.5-3 J/cm^2) using Sylvania GTE black light lamps (PUVA 4000, Waldmann/Germany; output 6.5 mw/cm^2). Absorption of UVA light by medium and the plastic cover of the culture plates was measured as described below and taken into account. After irradiation the 8-MOP containing medium was removed and replaced by fresh medium.

Measurement of ^{3}H-Thymidine Uptake and Cell Counts

After irradiation the cells were kept for 24 hr in the dark. ^{3}H-thymidine (^{3}H-TdR, spc act 5 Ci/mmol, TRA 120, Amersham Buchler, Braunschweig/Germany) was added to each culture corresponding to 2 μCi/culture (volume of culture medium: 1 ml). 2 hr later the cells were washed several times with saline, trypsinized and harvested on to glass-fiber filters by means of a multiple sample harvester (Titertec, Flow Laboratories, Bonn/Germany). The filter strips were dried for 30 min at 60°C, then placed in 5 ml scintillation fluid (6 gm Szintillator Butyl PBD (Ciba Geigy) plus 40% Toluene and 60% Methylglycol ad 1000 ml).

The radioactivity of ^{3}H-TdR incorporated into DNA was determined by a Packard Tricarb spectrometer. All experiments were done at least 2 times and run in quadruplicates each time. The standard deviations (SD) were calculated.

Measurement of UVA Absorbance

Plastic Linbro covers were used as filters in order to reduce the intensity of the incident UVA light. Between 320 nm to 400 nm the transmission of the covers increased linearly from 30% to 80.5% as measured with a Zeiss photometer PM QII at intervals of 10 nm. The filter plates changed the maximum of the emitted light energy from 350 nm to 354 nm and reduced the quantity of UVB light from 0.8% to 0.2%. The time necessary to irradiate the cell cultures with 1 J/cm^2 was raised from 300 sec (3.33×10^4 erg/cm^2 × sec) to 535 sec (1.87×10^4 erg/cm^2 × sec) for one filter plate and to 1031 sec (9.7×10^3 erg/cm^2 × sec) using 2 filter plates.

Cell Plating

Cells were treated with either 0.5 μg 8-MOP per ml followed by 1 J/cm^2 or 0.05 μg 8-MOP per ml and 3 J/cm^2 in the same way as described above. After this the cells were harvested and re-seeded into new culture vessels. In these experiments fetal calf serum (FCS, Gibco Bio-Cult, Glasgow/Scotland) instead of calf serum was used. The cell number (determined in a Coulter counter) as well as the uptake of ^{3}H-TdR were assessed at daily intervals. All cultures were checked daily by phase contrast microscopy (Diavert, Leitz, Wetzlar/Germany). Untreated controls were run at the same time.

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Reprint requests to: Dr. Enno Christophers, Department of Dermatology, University of Kiel, 23 Kiel, Schittenhelmstr, 7 Deutschland.

Abbreviations:
DMSO: dimethylsulfoxide
^{3}H-TdR: ^{3}H-thymidine
8-MOP: 8-methoxypsoralen

RESULTS

Incubation of fibroblasts at the stage of confluency with varying concentrations of 8-MOP (1×10^{-4} to 1.0 μg/ml) for 1 hr showed no effect upon subsequent ^{3}H-TdR incorporation (Table I). Also, no effect was seen when the cultures were irradiated in the absence 8-MOP. Neither the cell number nor the ^{3}H-TdR incorporation rates were found to be significantly changed (Table I). As shown in Table II a significant decrease of the number of adherent cells was present after treatment with 1 μg 8-MOP per ml and 3 J/cm^2. Smaller concentrations (from 1×10^{-4} to 1×10^{-1} μg/ml) as well as less light (1 J/cm^2, 0.5 J/cm^2) showed no influence on cellular adherence.

In Fig 1 the ^{3}H-TdR incorporation rates are shown after treatment of the cells with different concentrations of 8-MOP followed by UVA irradiation with 1 and 3 J/cm^2. With 1 J/cm^2 ^{3}H-TdR uptake decreased linearly, when the 8-MOP concentrations were plotted semilogarithmically. A significant inhibition was present with 0.01 μg 8-MOP per ml and no incorporation take place at 10 μg/ml (Fig 1). Cells treated while in their stationary growth phase appeared to be more sensitive as compared to growing cells. In both stages (confluency and growth) the rate of inhibition decreased linearly between 3 orders of magnitude of the concentration of 8-MOP.

When cells were irradiated with 3 J/cm^2 during the logphase of growth, the slope of the curve remained as before. However, as compared to cultures treated with 1 J/cm^2 similar rates of inhibition were produced by a 10 times lower concentration of 8-MOP.

In separate experiments changes of the lengths of irradiation (from 300 to 1031 sec, Fig 2) did not reveal significant differences. Cells incubated with 1 μg 8-MOP per ml and irradiated with 1 J/cm^2 for different lengths of time showed similar decreases of ^{3}H-TdR incorporation.

As can be seen from Fig 1 the inhibition of ^{3}H-TdR uptake was approximately 50% in cultures treated with either 0.5 μg/ml and 1 J/cm^2 or 0.05 μg/ml and 3 J/cm^2. When the cells were reseeded after these 2 treatment modalities (0.5 μg 8-MOP per ml and 1 J/cm^2 or 0.05 μg 8-MOP and 3 J/cm^2) were applied an identical rate of plating (70%) was seen (Fig 3*a*). In the untreated controls the plating rate amounted to 78% (Fig 3*a*). The number of untreated cells which had plated doubled within 3 days and reached confluency, while the number of treated fibroblasts increased from 1.7×10^5 on day 1 to 2.5×10^5 cells per culture on day 4. This indicates that 47% of the plated fibroblasts had doubled within the 3 days. No major change was present on the following days (Fig 3*a*). Also, no significant difference was seen between the 2 modalities of treatment.

The incorporation of ^{3}H-TdR, which was measured in the same cultures, showed a steep rise between day 1 and day 2 after seeding. This was 2.6 times higher in the untreated controls as compared to treated fibroblasts. In the latter the peak in radioactivity measured on day 2 was followed by a smaller peak on day 5 (Fig 3*b*). As before no differences were found between the 2 treatment regimens used (0.5 μg/ml, 1 J/cm^2 or 0.05 μg/ml, 3 J/cm^2).

Inspection of the cultures by phase microscopy revealed no detectable differences in cell morphology between treated cells and controls.

DISCUSSION

In bacteria, viruses, yeasts and cultured mammalian cells when photosensitized by furocoumarins (psoralens) subsequent irradiation with UVA produced lethal or mutagenic effects [8–10,15–21]. This cellular damage appears to be explained by a photoreaction between psoralens and nucleic acids, which

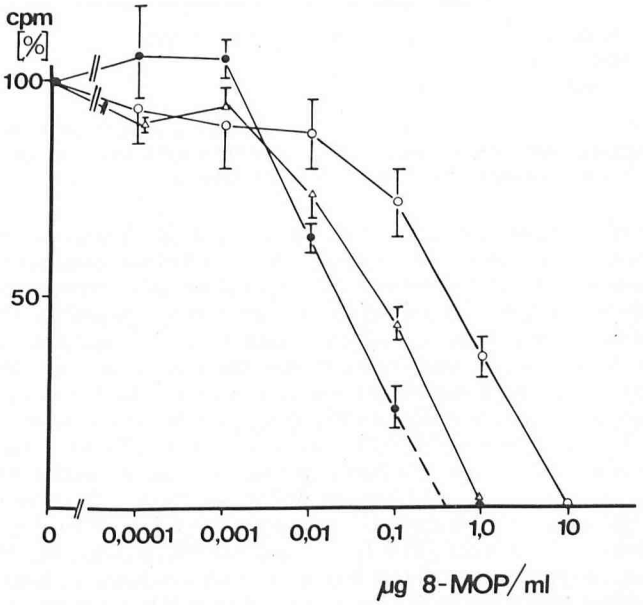

FIG 1. Dose-response curves of various concentrations of 8-methoxypsoralen followed by UVA (○ 1 J/cm^2, ● 3 J/cm^2) on the mean ± SD ^{3}H-thymidine-incorporation in growing fibroblast cultures (quadruplicates). For comparison cultures in stationary phase of growth (△) treated with 1 J/cm^2 are recorded.

TABLE I. *^{3}H-Thymidine (^{3}H-TdR) incorporation in confluent fibroblast cultures treated with 8-methoxypsoralen (8-MOP) or UVA*

8-MOP (μg/ml)	0	0.0001	0.001	0.01	0.1	1
^{3}H-TdR incorporation (cpm ± SD)	9944 ± 923	9959 ± 1489	10071 ± 499	9693 ± 788	9779 ± 1008	9763 ± 1322
UVA (J/cm^2)[a]	0	1	2	3	4	5
^{3}H-TdR incorporation (cpm ± SD)	18896 ± 2138	17802 ± 2329	16163 ± 2243	17402 ± 4038	16850 ± 3120	14303 ± 3167
Cell counts (n × 10^{-5} ± SD)	4.1 ± 0.1	4.1 ± 0.3	4.2 ± 0.3	4.3 ± 0.3	4.3 ± 0.4	4.0 ± 0.3

[a] The 2 experiments were performed separately thus the ^{3}H-TdR incorporation rates cannot be compared.

TABLE II. *Number of adherent fibroblasts (n × 10^{-5} ± SD) per culture 24 hr after in vitro PUVA-treatment (age of culture: 5 days, logarithmic growth phase)*

8-MOP (μg/ml) / UVA (J/cm^2)	Before treatment	0	0.0001	0.001	0.01	0.1	1
0.5	1.5 ± 0.2	2.9 ± 0.2	3 ± 0.1	3 ± 0.1	3.1 ± 0.3	2.7 ± 0.3	2.3 ± 0.3
1	0.7 ± 0.1	1.7 ± 0.2	1.8 ± 0.1	2 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.4 ± 0.2
3	2.0 ± 0.2	3.3 ± 0.2	3.5 ± 0.1	3.5 ± 0.2	3.2 ± 0.1	2.4 ± 0.3	1 ± 0.2

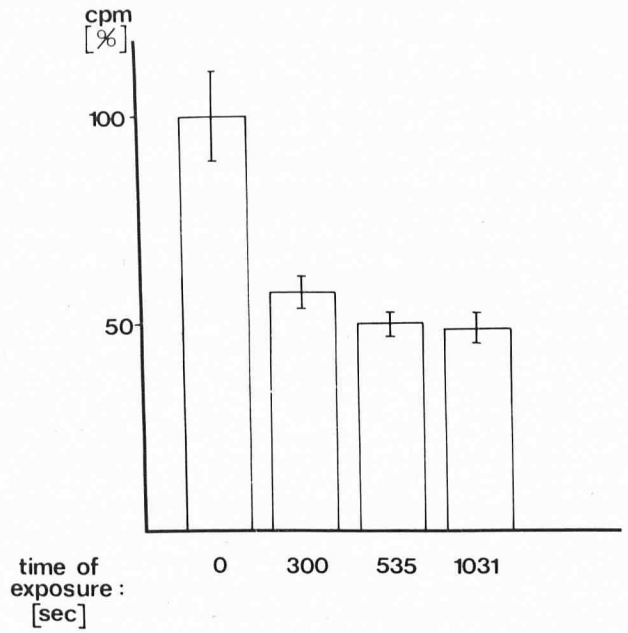

FIG 2. Effect of length of UVA irradiation (1 J/cm^2) on growing fibroblast cultures pretreated with 1 μg 8-methoxypsoralen per ml for 1 hr. Data represent means ± SD of quadruplicates.

occurs both *in vivo* and *in vitro* [8–10,12,14–21]. Various experiments have been performed in order to further characterize these effects on the growth of mammalian cells. Among such factors as temperature, experimental timing, growth media, photobinding capacity of the applied photosensitizers etc [8–10,15,16,18,19] their dosages and the energy of UVA light appear to play a significant role [10,12,20–26]. Baden et al [9] treated human fibroblasts with 50 μg/ml 8-MOP and observed a 90% inhibition of ^{3}H-TdR uptake after the cells were irradiated with 0.27 J/cm^2. Walter et al [13] by using trioxsalen (0.43 μg/ml and 0.25 J/cm^2 UVA) in epidermal organ cultures found a decrease of epidermal DNA synthesis by 70%. With higher doses of UVA light (10.8 J/cm^2) Bordin, Baccichetti, and Musajo [10] found a 52% inhibition of DNA synthesis in Ehrlich ascites cells after exposure to 2.4–3.6 μg 8-MOP per ml. Also, Trosko and Isoun [8] using human amnion cells incubated with 2×10^{-5} M 4,5′,8-trimethylpsoralen (approximately 4 μg/ml) found a complete inhibition of DNA synthesis when irradiated with less than 0.5 J/cm^2 UVA.

The dosages of 8-MOP used in the present study (10^{-4}–10 μg/ml) include the observed range of serum concentrations [27] of 8-MOP after oral uptake of therapeutical doses. When used alone, neither the applied concentrations of 8-MOP nor the different dosages of UVA showed a substantial effect. On the other hand, when the incubation with 8-MOP was followed by UVA irradiation, the incorporation of ^{3}H-TdR became markedly inhibited. These effects were seen after 24 hr whereas with our present dose-regimens no inhibition of ^{3}H-TdR uptake was measured immediately after treatment (data not presented).

Using 2 dosages of UVA light (1 J/cm^2 and 3 J/cm^2) an increasing inhibition of DNA synthesis was produced over a range of approximately 3 orders of magnitude of the 8-MOP concentration. Also, nearly the same degree of inhibition as obtained with 1 J/cm^2 was present when cultures treated with concentrations of 8-MOP 10 times lower were irradiated with 3 J/cm^2 UVA. This indicates that changes in the dose of UVA applied to cells photosensitized with 8-MOP are of stronger influence as compared to changes in the concentration of 8-MOP used for photosensitization. The possibility that the length of irradiation may potentiate the effect of treatment can be excluded by our experimental data (Fig 2).

The disproportionate influence of light appears to be confirmed by the plating experiments. Again, cells treated with either 0.05 μg/ml 8-MOP and 3 J/cm^2 showed the same growth characteristics as compared to those treated with 0.5 μg/ml 8-MOP and 1 J/cm^2 (Fig 3). Although this finding cannot be adequately explained at the moment, it correlates with our clinical experience, that changes in the energy of UVA are known to be of more importance as compared to the blood levels of 8-MOP. The latter are reported to vary considerably from patient to patient [27].

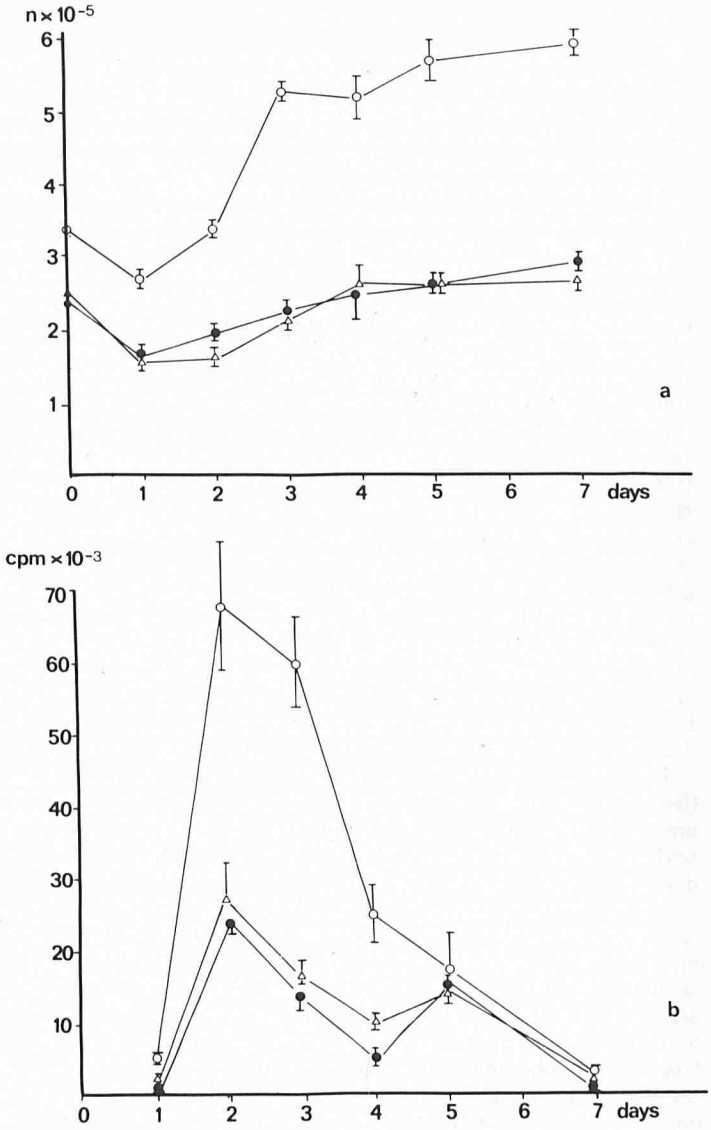

FIG 3. Cell number (*a*) and ^{3}H-thymidine incorporation (*b*) of fibroblast cultures which were trypsinized and re-seeded after treatment with 0.5 μg + 1 J/cm^2 (●) or 0.05 μg + 3 J/cm^2 (△). Controls (○) as well as treated cultures were done in quadruplicates, data represent means ± SD. On day 0 the total number of seeded cells is recorded (*a*), whereas on all subsequent days only cells firmly attached to substratum are counted.

Comparison of the number of cells adhering to the substratum with the incorporation rates of ^{3}H-TdR after PUVA treatment reveals that the cell number is beginning to be affected at dosages which reduce the incorporated radioactivity to zero (Fig 1 and Table II). At lower dosages of light and drug, no effect on cell number is seen. Light microscopical examination revealed no structural alterations indicative of cytotoxic changes. Also, treatment of the cells with PUVA which inhibited DNA synthesis by 50% was not followed by a loss of cellular attachment. In fact these cells were able to establish

new contacts with the substratum at approximately the same rate as the controls. Thus those cells sublethally treated by PUVA appear to be unable to replicate but still retain the capacity to fulfill other functions such as spreading and attachment.

It is noteworthy that after inhibiting DNA synthesis by 50% a proportion of cells will undergo further divisions. Quantitatively this fraction corresponds to cells not being photoinactivated to replicate. Whether this is the case after applying varying dosages of PUVA will be a matter of further studies.

The mechanism by which PUVA treatment inhibits cellular replication is not entirely clear. Whereas interstrand cross-linking of DNA as produced by psoralens and UVA light is considered incompatible with cell division ultimately resulting in cellular death, sublethal photoinactivation could also take place via other mechanisms. In Ehrlich ascites cells Bordin, Baccichetti, and Musajo [10] observed a 40% inhibition of RNA synthesis after treatment of the cells with low dose PUVA. Heimer, Ben-Hur, and Riklis [28] recently demonstrated specific inhibition of protein synthesis in a XD cell line using dosages of 8-MOP and UVA which were similar to those used in this study.

In human neutrophil chemotaxis assays the oriented movement of cells was shown to be inhibited by low dose PUVA treatment [29]. Also repair mechanisms of nuclear DNA as observed by Baden et al [9] need consideration. Hence the therapeutic benefit of PUVA in different dermatoses such as psoriasis [1–4,6] mycosis fungoides [1,5,7] or urticaria pigmentosa [30] deserves further work as to the way by which cutaneous cells can become photoinactivated *in vivo.*

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